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L20B Cells Simplify Culture of Polioviruses From Clinical Samples

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Culture of polioviruses from clinical samples is the gold-standard method for virological surveillance in the world-wide initiative to eradicate wild-type polioviruses. Two poliovirus-sensitive cell lines of human origin were used originally by the laboratories of the World Health Organisation (WHO) global poliovirus network. However, the cell lines used, Hep2 and RD, also support cytopathic growth of a variety of non-poliovirus enteroviruses. This can make detection of polioviruses in samples with mixtures of viruses difficult and time consuming. The development of mouse cell lines that express the gene for the human cellular receptor for polioviruses allows selective poliovirus culture, because very few non-poliovirus enteroviruses grow in these murine cells. A WHO Collaborative Study was initiated to test one such cell line, L20B, and to compare under routine conditions the sensitivity and selectivity of L20B cells against RD and Hep2 cells. Five laboratories in countries endemic or recently endemic for wild polioviruses participated. A total of 425 samples were tested prospectively in all three cell lines and there was a clear and consistent trend for greater sensitivity for polioviruses in L20B cells. Overall, 148/160 polioviruses were detected in L20B cells compared with 89/160 in RD and 98/160 in Hep2. In part, this finding was due to detection in L20B cells of polioviruses from samples that also contained non-poliovirus enteroviruses in which the poliovirus was masked in RD or Hep2 cells. However, L20B cells were also significantly more sensitive for poliovirus than either RD or Hep2 cells in three of the five study laboratories. The L20B cells were completely selective for polioviruses, as 0/89 wild type non-poliovirus enteroviruses produced cytopathic effect in L20B cells. Finally, L20B cells provided a diagnosis of poliovirus infection in the same time as RD and Hep2 cells from samples that contained poliovirus only, but substantially more quickly for samples that contained another enterovirus. Taken together, these data indicate that L20B cells simplify primary diagnosis of poliovirus from clinical samples and as a result they have been introduced for routine use by laboratories of the WHO global poliovirus network. ***J. Med. Virol. 58:188–192, 1999.***

KEY WORDS: poliovirus; L20B cell line; Hep2 cell line; RD cell line; enterovirus

INTRODUCTION

L20B cells are mouse L cells transfected with the gene for the human cellular receptor for poliovirus [Mendelsohn et al., 1989]. Expression of the receptor at the cell surface renders L20B cells susceptible to infection with poliovirus and a typical cytopathic effect develops. As the cells are of murine origin, very few other human enteric viruses produce cytopathic infection in L20B cells [Pipkin et al., 1993]. This selectivity for polioviruses makes the cell line an attractive option for the network of virological laboratories established by the World Health Organisation (WHO) to conduct surveillance for wild polioviruses and to provide evidence for the certification of poliomyelitis eradication [Hull and Dowdle, 1997]. Originally, laboratories in the network used the continuous cell lines RD, derived from a human rhabdomyosarcoma, and Hep2, derived from a human epidermoid carcinoma, for virus isolation and characterisation because of their availability, ease of maintenance and proven sensitivity to infection by polioviruses and other enteroviruses. This WHO collaborative study was set-up to field test L20B cells in par-

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allel with RD and Hep2 cells for culture of polioviruses from clinical samples.

MATERIALS AND METHODS

Cell Lines and Culture Media

Confluent monolayers of L20B cells in flasks were supplied to participants from a master cell bank of mycoplasma-free cells established at the National Institute for Biological Standards and Control (NIBSC). Participants were requested to subculture these cells every 5–7 days to establish stocks for cryopreservation and for the study. The recommended media for growth and maintenance was bicarbonate-buffered Eagle's modified essential medium (MEM; Earles salt base) supplemented with 10% v/v or 2% v/v newborn (or foetal) calf serum, respectively.

RD and Hep2 cells were already in use routinely in each laboratory. These cell stocks had originated from official collections. The recommended media for growth and maintenance was biocarbonate-buffered Eagle's MEM (Earles salt base) supplemented with l-glutamine and 5% v/v or 2% v/v foetal calf serum, respectively [Expanded Programme on Immunisation, 1997].

Validation of L20B Cells in the Study Laboratories

Three preparations of poliovirus type 1 (Sabin) coded 95/602, 96/592, and 96/596 were prepared at NIBSC. They had titres of 7.0, 1.0, and 0.2 $TCID_{50}$/0.1 ml, respectively, when titrated in Hep2C cells, a subline of Hep2 cells recommended for titration of poliovirus (Sabin) serotypes [WHO, 1990]. They were supplied frozen in dry ice and used to validate participants' newly established L20B cells by testing 10 cell cultures per preparation. This procedure also gave participants an opportunity to become familiar with poliovirus cytopathic effect in L20B cells prior to inoculation of clinical samples.

Prospective Study of Clinical Samples

Virological testing of stool samples from cases of acute flaccid paralysis is standardised in the WHO laboratory network [Hull and Dowdle, 1997]. Faecal specimens are treated with chloroform and observed in cell culture (RD and Hep2) over two passages of 7–10 days each before being reported as negative. Isolates are identified by microneutralisation tests using standard polyclonal typing antisera [Expanded Programme on Immunisation, 1997]. For the prospective study, participants were requested to inoculate concurrently all stool samples received over a 3-month period or at least 100 consecutive samples, whichever was the sooner, into L20B cells as well as the standard RD and Hep2 cell lines. The procedure for culture in L20B cells was identical to RD and Hep2 cells. All enterovirus isolates were serotyped with standard poliovirus antisera on the cell line(s) of primary isolation by standard methods. Participants reported poliovirus isolates directly into the National EPI system as per usual. When an isolate contained an enterovirus that could not be neutralised with the poliovirus antisera, then the isolate was tested against non-poliovirus enterovirus antiserum pools. If a non-poliovirus enterovirus was identified in this test, a further test with specific neutralising antisera against the identified virus was then performed on the original isolate to exclude the presence of a poliovirus. Any viruses that could not be identified with this procedure were recorded as "unidentified enterovirus." Results were recorded on standard report forms.

Participants

Five study sites in areas of current or recent wild poliovirus circulation were selected. The participants were:

Dr. Tary Naguib, Egyptian Organisation for Biological and Vaccine Production (VACSERA), Cairo, Egypt.
Dr. J. M. Deshpande, Enterovirus Research Centre, Bombay, India.
Dr. K. K. Datta, National Institute of Communicable Diseases, Delhi, India.
Dr. Nalinin Ramamurth, King Institute of Preventive Medicine, Modras, India.
Dr. Hinda Triki, Institue Pasteur de Tunis, Tunis, Tunisia.

The laboratories are identified by code letters in this report and the coding does not necessarily correspond to the order listed above.

RESULTS

Establishment of L20B Cells in Study Laboratories

L20B cells were received in all five study laboratories in early August 1996. The cells were reported as easy to handle; easy to cryopreserve; and easy to resuscitate from liquid nitrogen storage. An established split ratio of 1 to 4 for routine subculture was reported from two laboratories and is the same as used at NIBSC. The other laboratories did not report split ratios. Data were reported from two laboratories on the validation samples that confirmed the sensitivity of L20B cells in these two study laboratories (data not shown). The other laboratories did not report data with the titrated poliovirus type 1 samples because of difficulties in transport of the samples to the study sites in time for the start of the study. Instead, they used local poliovirus (Sabin) stocks to validate that their cells would grow polioviruses.

Prospective Study Samples

A total of 425 samples were studied during the course of the prospective study. The viruses detected in these samples are shown in Table I. The majority of isolates were poliovirus type 1. Intratypic differentiation results were not reported. A substantial proportion (40/160, 25%) of samples positive for poliovirus contained other enterovirus(es) and the overall non-poliovirus isolation rate was 20% (89/425). The num-

TABLE I. Viruses Detected in Prospective Study Samples

Virus(es) detected in stool sample	Number of samples with indicated result in laboratory					Total for all laboratories
	A	B	C	D	E	
Polio 1 only	4	16	14	37	27	98
Polio 2 only	0	0	2	0	0	2
Polio 3 only	1	4	1	0	0	6
>1 Polio serotype	0	3	11	0	0	14
Polio 1, 2 or 3 + another enterovirus	0	14	11	9	6	40
Enterovirus(es) other than polio	3	37	3	5	1	49
None	85	27	40	49	15	216
Total numbers of samples studied	93	101	82	100	49	425
Study period						
Start	26 Aug 1996	18 Aug 1996	12 Aug 1996	19 Aug 1996	ng	
End	11 Dec 1996	3 Sep 1996	18 Dec 1996	29 Nov 1996	ng	

ng, not given.

bers and types of isolates varied considerably between laboratories as did the study period required to obtain approximately 100 samples. There were no reports of other enteric viruses (such as adeno- or reoviruses) in any cell line from any laboratory.

Sensitivity of Cell Lines for Isolation of Polioviruses

A total of 160 polioviruses were isolated from the study samples (Table I). The numbers of poliovirus isolates made in RD, Hep2, and L20B cell lines are shown in Table II. More polioviruses were isolated in L20B cells than the other cell lines. L20B cells were significantly more sensitive than RD and Hep2 in laboratory C; significantly more sensitive than Hep2 in laboratory D; and significantly more sensitive than RD in laboratory E. They were not significantly more sensitive than either RD or Hep2 in laboratories A and B, but more isolates were made in L20B cells in both laboratories, which is consistent with a trend for greater sensitivity. RD and Hep2 cells were of equivalent sensitivity in four of the five laboratories but in laboratory B, RD cells were markedly less sensitive for isolation of polioviruses.

The trend for increased sensitivity of L20B cells over RD and Hep2 may be due to a higher inherent sensitivity for polioviruses; or to improved detection of polioviruses in samples that contain other enteroviruses where the other enterovirus masks identification of the poliovirus; or for other reasons. Table III shows that 30/46 (64%) polioviruses detected only in L20B cells were from samples in which non-polio enterovirus were not isolated in concurrently inoculated RD or Hep2 cells, a finding that suggests, as no other reason was identified, that greater inherent sensitivity was a major factor in some study laboratories. The L20B cells allowed isolation of polioviruses from 16 samples, which also contained non-polio enteroviruses that otherwise would have been missed in RD or Hep2 cultures.

Despite the greater sensitivity of L20B cells, some isolates were missed in this cell line. There was no obvious clustering of poliovirus serotypes that did not grow in L20B cells (data not shown). Some isolates that failed to grow in L20B cells were from samples toxic to that cell line. Sample toxicity was therefore compared across all cell lines and all laboratories. Overall, only 5% (19/425) of samples were toxic in any cell line and L20B cells were no more sensitive to toxicity than RD cells, although both appeared slightly more sensitive than Hep2 (data not shown). The majority of toxic samples occurred in Laboratory E, where 27% (13/49) samples were toxic to one or more cell lines.

Selectivity of Cell Lines

Previous studies showed that prototype strains of a large number of non-poliovirus enteroviruses did not produce cytopathic growth in L20B cells [Pipkin et al., 1993]. An important question addressed in the current study was whether wild-type non-poliovirus enteroviruses would produce cytopathic effect or not. A total of 89 wild-type non-poliovirus enteroviruses were isolated during the prospective study (Table I) and Table IV shows that none had a cytopathic effect in L20B cells, thus confirming the selectivity of L20B cells for polioviruses under field conditions.

RD cells were overall more sensitive that Hep2 cells for culture of non-poliovirus enteroviruses (Table IV), the majority of which were unidentified, but Hep2 cells were more sensitive than RD cells for detection of coxsackie B viruses (19 vs. 1 isolate, respectively) (data not shown).

Timeliness of Poliovirus Detection

In addition to sensitivity and specificity of poliovirus detection, timeliness of diagnosis of poliovirus infection is an important programmatic consideration for the eradication initiative. The time to make a diagnosis of poliovirus was compared in RD, Hep2 and L20B cells for samples that contained poliovirus(es) only. A subset of data was used for this comparison because laboratory E did not report time to achieve a diagnosis. Polioviruses were identified in about the same time in RD, L20B, and Hep2 cells. A substantial increase in poliovirus isolation rate was seen between day 11 and

TABLE II. Sensitivity of Cell Lines for Isolation of Polioviruses From Prospective Study Samples

Cell line	Number of poliovirus isolates in the indicated cell line in laboratory					Total for all laboratories[a]
	A	B	C	D	E	
RD	3	18	22	37	9	89
Hep2	3	29	21	35	10	98
L20B	5[b]	33[b]	36[c]	44[d]	30[e]	148

[a]This Table shows the number of times a virus was isolated from each of the cell lines. As each sample was inoculated into all three cell lines and a virus was often isolated in more than one cell line, the total number of polioviruses in this table differs from that given in Table I.
[b]Number of isolates in L20B cells not significantly different from number of isolates in RD or Hep2 cells (McNemar test).
[c]Number of isolates in L20B cells significantly greater than number of isolates in RD and Hep2 cells ($P = .0005$ and .0007, respectively; McNemar test).
[d]Number of isolates in L20B cells significantly greater than number of isolates in Hep2 ($P = .004$, McNemar test) but not significantly different from number of isolates in RD cells (McNemar test).
[e]Number of isolates in L20B cells significantly greater than number of isolates in RD cells ($P = .002$, McNemar test) but not significantly different from number of isolates in Hep2 cells (McNemar test).

TABLE III. Results in RD and Hep2 Cells for Samples in Which Polioviruses Were Isolated Only in L20B Cells

Result in cell line			Number of samples with indicated result in laboratory					Total for all laboratories
RD	Hep2	L20B	A	B	C	D	E	
—[a]	—	Polio	2	1	7	8	12	30
Non-polio[b]	—	Polio	0	3	0	0	0	3
—	Non-polio	Polio	0	0	0	0	4	4
Non-polio	Non-polio	Polio	0	0	0	7	2	9

[a]—, no virus detected or sample toxic in that cell line.
[b]Non-polio, non-poliovirus enterovirus isolated in indicated cell line.

TABLE IV. Selectivity of Cell Lines for Isolation of Poliovirus From Prospective Study Samples

Cell line	Number of enteroviruses other than polio isolated in the indicated cell line in laboratory					Total for all laboratories[a]
	A	B	C	D	E	
RD	3	40	9	14	3	69
Hep2	1	13	6	14	7	41
L20B	0	0	0	0	0	0

[a]A virus was often isolated in more than one cell line, hence the non-poliovirus enteroviruses reported in this table differs from Table I.

12 in all cell lines after a blind passage between days 7 and 10. For samples that contained a poliovirus and another enterovirus, L20B cells achieved poliovirus identification substantially more quickly than either of the other cell lines (Fig. 1). For these samples, Hep2 cells were quicker than RD at identifying the poliovirus.

DISCUSSION

Research in the specialised laboratories of the WHO global poliovirus laboratory network suggested that mouse cell lines genetically engineered to express the human cellular receptor for poliovirus were highly selective for poliovirus detection [Pipkin et al., 1993; Hovi and Stenvik, 1994]. Five experienced laboratories from the network were chosen to field test the L20B cell line to determine whether this promise would be realised under routine conditions of use. The results of the study show that L20B cells can provide greater sensitivity and specificity for detection of polioviruses from clinical samples. The study also showed that L20B cells can provide a quicker diagnosis of poliovirus infection especially in samples that contain another enterovirus.

The trend for improved sensitivity of L20B cells compared with RD or Hep2 cells was due in part to the selectivity of L20B cells to grow poliovirus from samples that contained other enteroviruses. In RD and Hep2 cells, polioviruses were sometimes masked by the concurrent growth of the other enteroviruses. However, an unexpected finding was that the L20B cells were significantly more sensitive than the RD and Hep2 cells in routine use in some, but not all, of the study laboratories. Cross contamination of L20B cells with polioviruses seems an unlikely explanation for this finding, because no viruses were detected in many samples in these laboratories. Suboptimal sensitivity of the RD and Hep2 cells may be a possible explanation. One implication of this observation may be that tests of poliovirus sensitivity, for example, regular assays of standards of known titre, should be reinforced as an important part of the quality assurance system for the laboratory network. Another implication may be

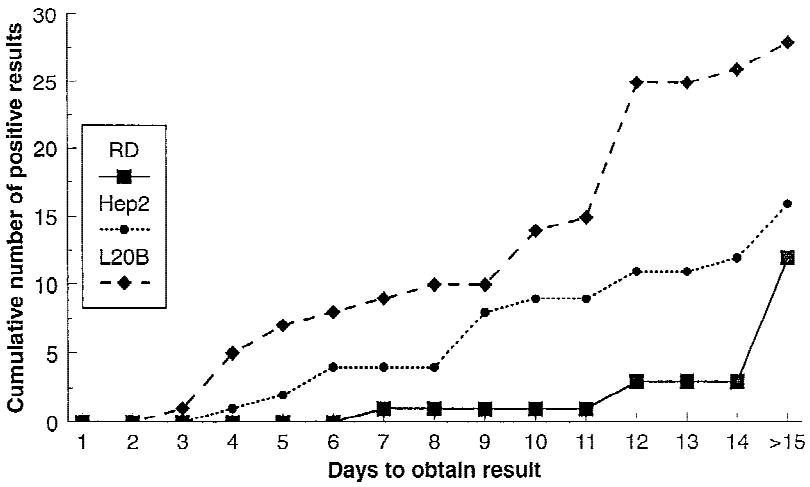

Fig. 1. Time to achieve poliovirus results for samples that contained poliovirus and another enterovirus.

that additional cell lines, such as primary monkey kidney or a continuous monkey kidney cell line such as Vero, warrant consideration for use in some circumstances.

The polioviruses detected in this study were predominantly poliovirus type 1. Although polioviruses types 2 and 3 were under-represented in the study experience of culture of more than 800 poliovirus isolates in L20B cells at NIBSC (unpublished data) shows that poliovirus 1, 2, and 3 of both Sabin and wild-type origin grow equally well in these cells.

Some poliovirus isolates were missed in L20B cells. Although operational or stochastic explanations are most likely investigations to exclude a biological cause should be considered on such specimens. Reinoculation of the original isolate into L20B plus the cell line originally virus positive would be the simplest test. Any polioviruses that consistently failed to grow in L20B cells would be of considerable interest.

The selectivity of L20B cells for polioviruses was confirmed in this study by the failure of a large number of wild-type non-poliovirus enteroviruses to produce cytopathic effect in L20B cells. The cells are not absolutely specific for polioviruses, however, because some other human enteric viruses, such as wild-type reoviruses, will grow in both L20B cells and the parental L cell from which they are derived. However, this lack of specificity was clearly not a problem in this field test of the cells.

The primary activities of the WHO global poliovirus network are poliovirus detection and characterisation [Hull and Dowdle, 1997]. This study shows that introduction of mouse cells expressing the human cellular receptor for poliovirus into the network will simplify these activities. Standard procedures and reagents are required for this purpose. The L20B cells used in this study were from a cell bank established at NIBSC to serve as a standard source. Cells from this bank have been distributed to regional reference laboratories for establishment of secondary cell banks and distribution onward to all laboratories of the network. There is no evidence from long-term (longer than 1 year) passage at NIBSC that L20B cells loose sensitivity due to the theoretical problem of outgrowth of receptor negative cells.

Recommended practice, as with all cell lines, should be to return to fresh stocks from liquid nitrogen at defined intervals (e.g., 6 months) to minimise mycoplasma contamination problems. One final advantage of the L20B cell line used in this study is the use of a medium for growth and maintenance similar to the medium already used by network laboratories for growth of their other cell lines.

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